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Aluminum mediates compositional alterations of polar lipid classes in maize seedlings

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Abstract

Changes in lipid composition were investigated on maize roots and shoots under aluminum stress. After 4 d exposure to 100 μM Al, root growth was inhibited while shoot growth was not affected. In roots, the decrease of the DBI (double bond index) of total fatty acids may signal a decrease in membrane fluidity. The total lipids (TL) decreased by 49%, but phospholipids (PL), phosphatidylcholine (PC) and phosphatidylinositol (PI) increased to approximately 3-fold. The MGDG increased to 2-fold but no significant change was found in the DGDG. The steryl lipids (SL) increased by 69%. The SL/PL ratio decreased from 2.64 to 1.52 and the MGDG/DGDG ratio increased from 0.45 to 1.06 in roots of Al-stressed plants. Al leads to oxidative stress in roots of treated plants as indicated by the increase of malondialdehyde (MDA) concentrations. In shoots, changes in fatty acid composition were associated with an increase of the DBI in all lipid classes except that of the DGDG decreased. The PG was the lipid class which shows the large variation of fatty acid composition. No significant changes were found either for TL, PL, SL or MDA concentrations in shoots of Al-treated plants. While PE levels did not show significant change, PI and PG increased and PC decreased. However, the Al caused 87% decrease in the GL levels. The MGDG and DGDG decreased to 19- and 8-fold, respectively.

The deleterious effects of Al on polar lipids could be caused by a direct intervention of Al on plasma membrane and/or alteration of cell metabolism.

Keywords: *Zea mays* L.; Aluminum toxicity; Lipid peroxidation; Lipids; Fatty acids; Glycolipids; Phospholipids; Steryl lipids

1. Introduction

A wide range of cellular responses occur when plants are exposed to a variety of environmental stresses such as freezing, drought, salinity and metal toxicity. The cells tend to respond to disturbing environmental factors by altering membrane lipid composition, and such changes are thought to restore optimal physical properties (Thompson, 1992). The plasma membrane has been suggested to be an efficient barrier preventing metals from entering the symplasm (Taylor, 1988a; Bennet and Breen, 1991a,b). Heavy metals act as factors that induce compositional changes of membrane lipids (Harwood, 1984). Regulation of lipid membrane composition and adjustment of the unsaturation level of the membrane fatty acids are extremely important to deal with metal toxicity and make a considerable

Abbreviations: DGDG, digalactosyl–diacylglycerol; GL, glycolipids; MDA, malondialdehyde; MGDG, monogalactosyl–diacylglycerol; PC, phosphatidylcholine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipids; SL, steryl lipids; TL, total lipids.

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contribution to plant survival. Changes in the properties of cellular membranes occur to ensure the proper function of processes that take place within them, and lead to ameliorative growth contributing to plant adaptation. Aluminum is the most abundant element of the earth crust, representing about 7% of its mass. It can be one of the most growth-limiting factor (Foy, 1988), possibly affecting approx. 40% of the world's arable land that is potentially usable for food and biomass production (Ma et al., 2001). Toxicity of Al concerns, however, only some of its soluble forms, where the most toxic monomer species Al^{3+} prevails in acidic conditions (Fageria et al., 1988; Kinraide and Parker, 1989; Delhaize and Ryan, 1995). Inhibition of root growth is well known effect of Al toxicity and root tips has been suggested as a primary site for Al-induced injury in plants (Ryan et al., 1993). Aluminum affects many aspects of physiology, biochemistry and molecular biology of the cell by disrupting a numerous components such as membrane lipids. It has been shown that Al can cause alterations of plasma membrane properties by binding to membrane lipids or proteins (Vierstra and Haug, 1978). Changes of the membrane lipid architecture induced by Al can lead to modification of membrane permeability (Zhao et al., 1987; Gunsé et al., 1997). However, the effects of Al on membrane lipids are less commonly studied. This work was thus focused on changes in polar lipids of maize roots and shoots in response to Al stress.

2. Results and discussion

2.1. Effect of aluminum on growth

Exposure of maize plants to 100 μM Al leads to a number of structural and morphological alterations occurring early on the root system. These responses include decreases in root elongation of individual roots and extent of lateral roots. This shortening is associated with thickening of primary and secondary laterals. In addition, several visible criteria of Al toxicity can be observed, involving swelling, curving and browning of the root apices. Al significantly decreased the f. wt. and d. wt. of roots by 46.3% and 58.5%, respectively (Fig. 1(a,c)). However, Al had no effect on shoot growth (Fig. 1(b,d)). Numerous studies have described morphological changes in roots under Al stress (Taylor, 1988a,b; Bennet and Breen, 1991a,b). It is generally assumed that root elongation is the best measure for Al toxicity symptoms (Ryan et al., 1992). It has been suggested that inhibition of root growth induced by Al may result from disturbance of the cell divisions in the root meristematic zone (Doncheva et al., 2005).

2.2. Aluminum-induced alterations of root lipids

Changes in the relative abundance of the major fatty acid species in roots were plotted and are shown in

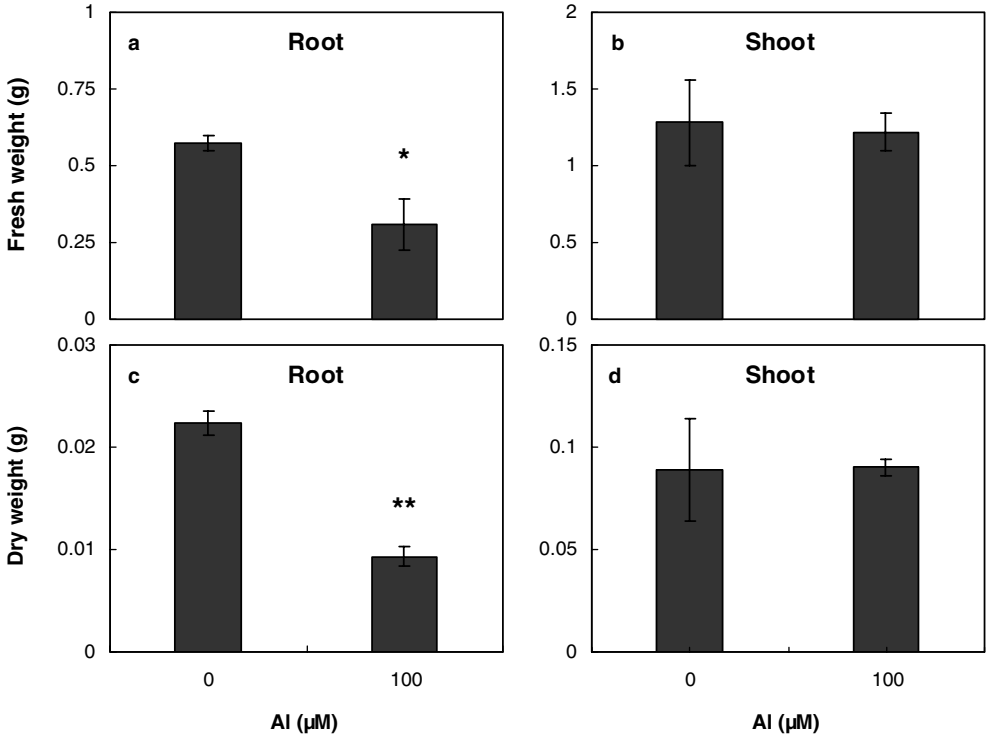

Fig. 1. Fresh and dry weight of maize roots (a,c) and shoots (b,d) at 0 and 100 μM Al. Maize seedlings were grown in hydroponics for 8 days then treated with Al for 4 days. The results are given as the means ± SE of at least five independent experiments. Significant differences between treated and control plants were determined using one-way ANOVA: $^{*}P < 0.05$; $^{**}P < 0.01$.

Fig. 2. Exposure of maize plants to 100 μM Al caused a significantly lower proportion of saturated palmitic acid (16:0) and monounsaturated oleic acid (18:1) than was observed in the control seedlings, while the proportions of other fatty acids did not differ from the control. The level of 18:1 decreased from 13.7 to 6.1 mol% of total fatty acids and that of 16:0 from 25.7 to 18.7 mol% of total fatty acids. To provide an estimation of the membrane unsaturation level, the double bond index (DBI) was calculated for 18 C fatty acids from the mol% fatty acid data. The double bond index of total lipids (TL) from roots decreased in treated seedlings (Table 1). Thus, we suggest that alteration of the fatty acid unsaturation level in roots may lead to reduction of membrane lipid fluidity. These observations are similar to the results of previous studies in maize (Suhayda and Haug, 1986), in Al-sensitive sorghum cultivar (Peixoto et al., 2001), in Al-sensitive fungus *Amanita muscaria* (Zel et al., 1993b) and in *Thermoplasma acidophilum* (Vierstra and Haug, 1978). Al binding to membrane phospholipids has been suggested as one of the toxic lesions induced by Al and resulted in decreased membrane fluidity (Vierstra and Haug, 1978; Deleers et al., 1985; Shi and Haug, 1988; Akeson et al., 1989). By contrast, it has been shown that Al-induced an increase or a lower reduction in the membrane fluidity of an Al-tolerant sorghum cultivar (Peixoto et al., 2001) and an increase in that of an Al-resistant fungus *Lactarius piperatus* (Zel et al., 1993a). A less unsaturated fatty acid composition

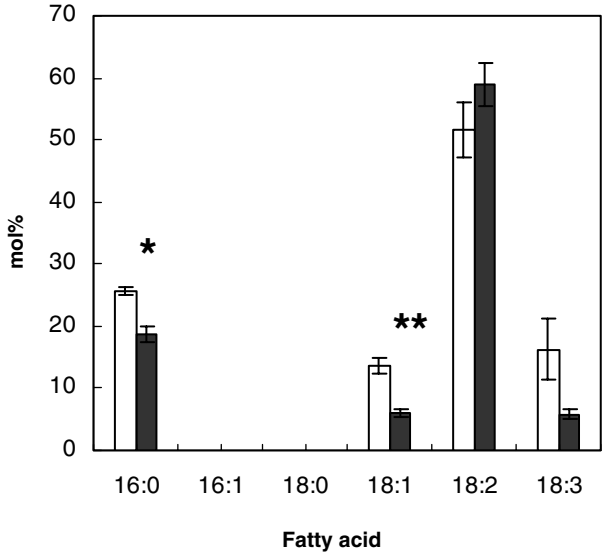

Fig. 2. Total fatty acid composition of maize roots at 0 and 100 μM Al. Maize seedlings were grown in hydroponics for 8 days then treated with Al for 4 days. The histograms presented mean values ± SE obtained from three independent experiments. Significant differences between Al-treated (black bars) and control plants (white bars) were determined using one-way ANOVA: $^{*}P < 0.05$; $^{**}P < 0.01$.

Table 1
Double bond indices calculated for each root lipid class of maize grown at 0 and 100 μM Al

Lipids	Al (μM)	
	0	100
TL	1.66	1.41
PI	2.12	2.02
PC	0.62	0.74
MGDG	0.90	1.52
DGDG	1.93	1.16

The double bond index was estimated from mol% fatty acid values indicated in Figs. 2 and 3.

may be a response that may have important role in limiting the entry of toxic Al ions and thus enabling the plant to continue growth under Al stress. The decrease in the unsaturation level may be the consequence of decreased or inhibition of desaturases activities. To evaluate measurable effects of Al on lipid composition, phospholipid and glycolipid class composition was also determined. Examination of Fig. 3 reveals several changes in the fatty acid profiles of the glycolipids in response to Al, while no significant changes were detected in those of PC and PI. The 16:0 and 18:3 levels of MGDG increased, while that of 16:1 decreased. However, there were increases in 16:0, 16:1 and 18:0 levels of the DGDG with a concomitant decrease in diunsaturated 18:2 and triunsaturated 18:3 fatty acids (Fig. 3). The double bond indices decreased in fatty acids from PI and DGDG and increased in those from PC and MGDG under Al stress (Table 1). Total lipid content, as expressed by mg [g d. wt.]$^{-1}$, showed a significant decrease by 49.1% in treated seedlings (Table 2). However, with regard to different lipid classes, the phospholipid (PL) content increased to 3-fold, while glycolipids (GL) did no show significant response to Al (Table 2). The PI and PC were markedly increased to about 3-fold in treated seedlings (Table 3). These changes may represent an important mechanism to mediate the response to Al. Thus, the PI and PC could be preferred species of Al-injured membranes. In plasma membranes isolated from entire roots of Al-tolerant (PT741) cultivar of wheat, PL is not affected by Al, but the PC concentration increased after exposure to 20 μM Al (Zhang et al., 1997). Lindberg and Griffiths (1993) have observed increases in the relative abundance of PC in plasma membranes from roots of *Beta vulgaris*. By contrast, Zhang et al. (1996) have shown that prolonged exposure for 3 days to 50 μM Al decreased the PL and PC content in the microsomal membranes purified from 5-mm root tips of two wheat cultivars. Enhanced abundance of PC is involved in producing less packed membranes and as a consequence increased membrane fluidity (Shinitzky, 1984). Our results show that the total steryl lipids (SL) significantly increased

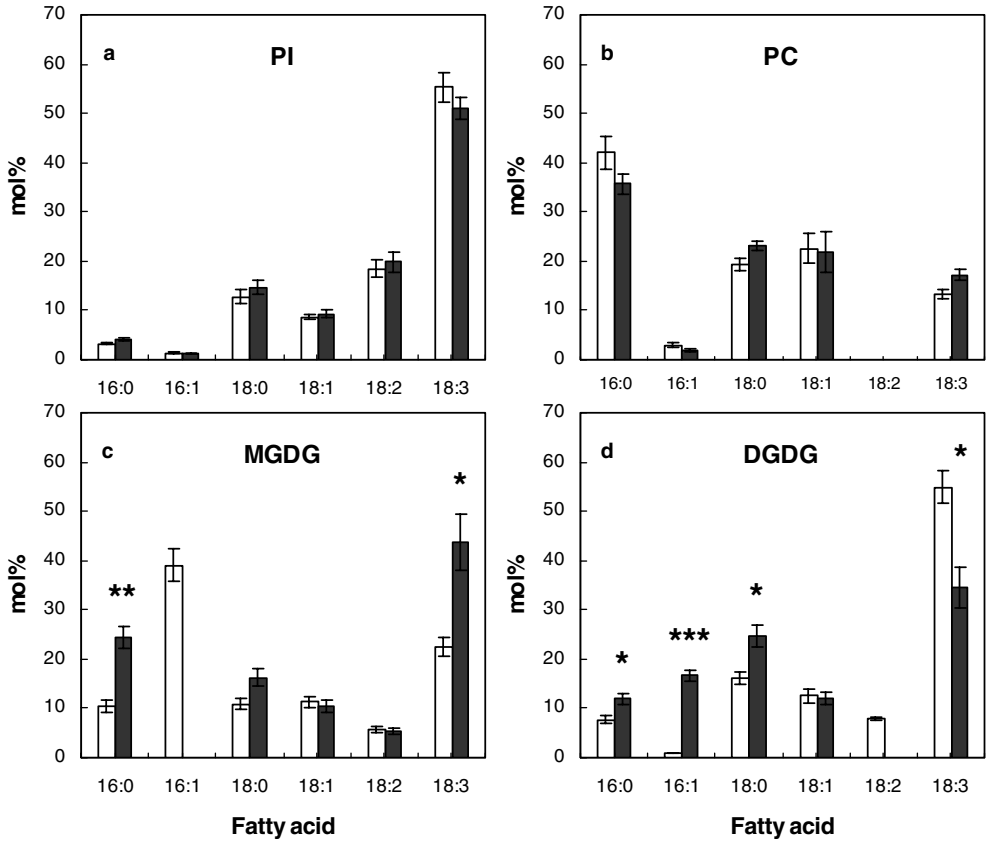

Fig. 3. Effect of Al on fatty acid profiles of various lipid classes obtained from maize roots. The maize (*Zea mays* L.) seedlings were grown in hydroponics for 8 days then treated with 100 μM Al for 4 days. The histograms presented mean values ± SE obtained from three independent experiments. Significant differences between Al-treated (black bars) and control plants (white bars) were determined using one-way ANOVA: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Table 2
Aluminum effect on lipids classes of maize roots

Al (μM)	Lipids (mg g^{-1} d. wt.)				SL/PL
	Total lipids	Phospholipids	Glycolipids	Steryl lipids	
0	14.23 ± 1.25	0.82 ± 0.08	1.12 ± 0.10	2.17 ± 0.21	2.64
100	7.24 ± 1.73*	2.40 ± 0.14***	1.46 ± 0.09	3.66 ± 0.28*	1.52

Values represent means ± SE ($n = 3$). Significant differences between treated and control plants were determined using one-way ANOVA.
* $P < 0.05$.
*** $P < 0.001$.

Table 3
Aluminum effect on different lipid classes obtained from maize roots

Al (μM)	Lipid classes (mg g^{-1} d. wt.)				MGDG/DGDG
	PI	PC	MGDG	DGDG	
0	0.63 ± 0.11	0.19 ± 0.02	0.35 ± 0.05	0.77 ± 0.10	0.45
100	1.88 ± 0.18**	0.52 ± 0.07*	0.75 ± 0.03**	0.71 ± 0.09	1.06

Eight-day-old seedlings were grown in hydroponics and treated with 100 μM Al for 4 days. Values represent means ± SE ($n = 3$). Significant differences between treated and control plants were determined using one-way ANOVA.
* $P < 0.05$.
** $P < 0.01$.

by 68.7% followed by the decrease of SL/PL ratio after Al treatment (Table 2). The increase in SL was similar to other reports for other plant species *Secale cereale* (Lynch and Steponkus, 1987) and *Solanum tuberosum* (Palta et al., 1993) in response to cold acclimation. Zhang et al. (1997) showed that Al tolerance was accompanied by decreased SL content and decreased SL/PL ratio in root plasma membranes of an Al-tolerant wheat cultivar. In a previous study, however, they have indicated increased SL/PL ratio in microsomal membranes in both wheat genotypes (Zhang et al., 1996). While, the MGDG is accumulated to higher levels in roots of treated plants than in the control, the DGDG did not show any alteration (Table 3). Also, Al considerably increased (1.4-fold) the MGDG/DGDG ratio (Table 3). Aluminum increased MGDG/DGDG ratio in plasma membranes purified from entire roots (Zhang et al., 1997) and from root tips of the Al-tolerant wheat cultivar (Zhang et al., 1996). The salt resistance has been shown to be associated to higher MGDG/DGDG ratio in *Glycin max* (Zenoff et al., 1994). The DGDG levels can affect the membrane permeability. It has been reported that membrane vesicles containing DGDG were more permeable to Rb^+ than composed of PC (Webb and Green, 1989). Thus, maintaining lower DGDG levels can limit the uptake of toxic Al species and prevent Al-induced increase in membrane leakiness (Sasaki et al., 1994). There is evidence that Al triggers alterations of the biochemical and physiological properties of membrane lipids, thereby inducing change in membrane fluidity (Vierstra and Haug, 1978; Zel et al., 1993a,b). The MDA measurement might be the indicator to assess lipid peroxidation. In roots of Al-treated plants, the MDA concentration was increased by 32.6% (Fig. 4(a)). This may indicate the occurrence of lipid peroxidation. The decrease of the amount of TL in treated roots observed in this study may reflect a degradation process that lead to the accumulation of MDA. Environmental stresses are thought to affect membrane lipids leading to lipid peroxidation. Previous study on the water-deficit stress has shown an increase in MDA concentrations which is associated with a decrease in polyunsaturated fatty acids (PUFAs) (Ferrari-Iliou et al., 1992). Aluminum can modify lipid arrangement of the membrane facilitating lipid peroxidation by iron (II) (Gutteridge et al., 1985). Al treatment decreased linolenic acid in purified plasma membrane fraction of root apices of Al-sensitive *Sorghum* which is indicative of lipid peroxidation (Peixoto et al., 2001). Al significantly increased the MDA concentrations in plasma membranes of root apices of the Al-sensitive sorghum cultivar with no change in the Al-tolerant cultivar. By contrast, Al did not induce changes in MDA in plasma membranes of whole roots in sorghum cultivars (Peixoto et al., 2001) and other plant species (Heath and Packer, 1968; Gutteridge et al., 1985; Cakmak and Horst, 1991).

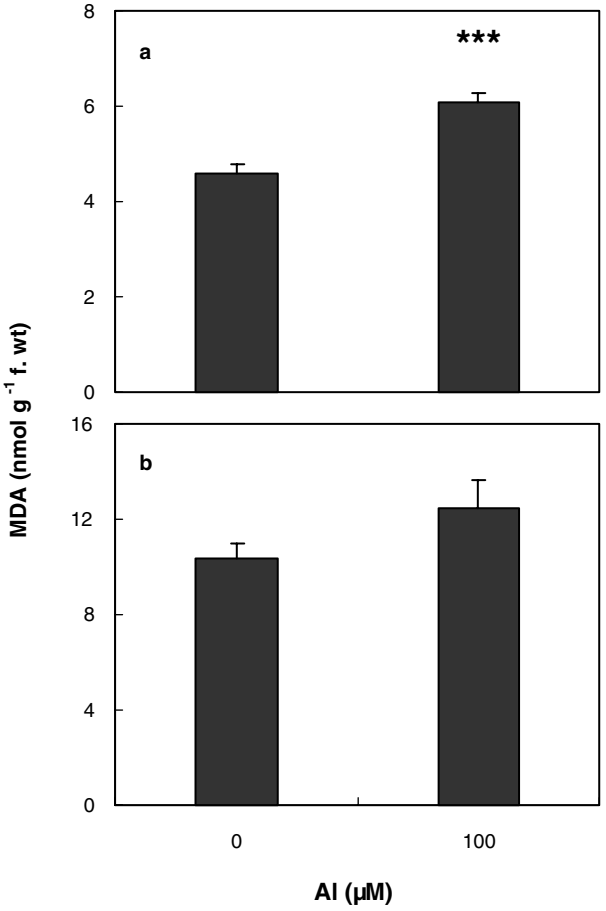

Fig. 4. Malondialdehyde (MDA) accumulation in roots (a) and shoots (b) of maize plants grown in hydroponics for 8 days then treated with 100 μM Al for 4 days. The results presented are the mean values ± SE obtained from at least five independent experiments. Significant differences between treated and control plants were determined using one-way ANOVA: $^{***}P < 0.001$.

2.3. Aluminum-induced alterations of shoot lipids

The fatty acid composition was also evaluated in shoots after Al treatment. The fatty acid profiles showed no detectable changes except that of 16:0 which exhibited significant increase from 13.6 to 16.2 mol% (Fig. 5). The double bond index showed a slight increase in plants grown under Al stress (Table 4). Several clear alterations are evident in fatty acid composition of individual class lipids of maize shoots in response to Al. Among the individual lipid classes, the principal changes apparent in response to Al were a decrease in the abundance of saturated 16:0 (PI, PG and PE), monounsaturated 16:1 (PG) and 18:1 (PI and PC) and a pronounced increase in polyunsaturated species, 18:2 (PI and PE) and 18:3 (PC and PG) (Fig. 6). Aluminum significantly decreased the proportion of 16:0 in the MGDG from

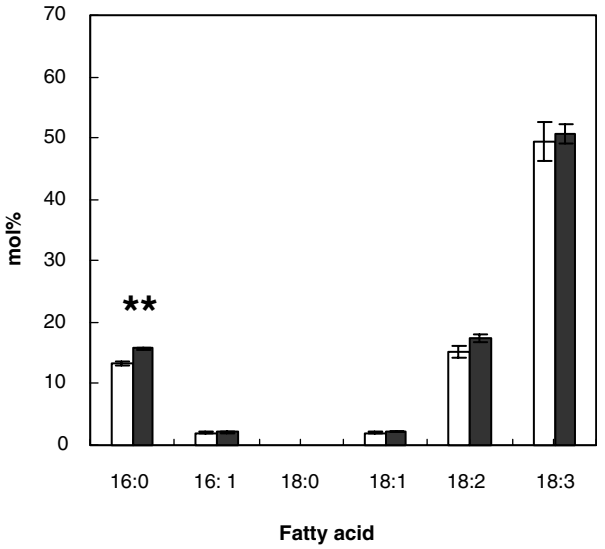

Fig. 5. Total fatty acid composition of maize shoots at 0 and 100 μM Al. The seedlings were grown in hydroponics for 8 days then treated with 100 μM Al for 4 days. The histograms presented mean values ± SE obtained from three independent experiments. Significant differences between Al-treated (black bars) and control plants (white bars) were determined using one-way ANOVA: $^{**}P < 0.01$.

Table 4
Double bond indices calculated for each shoot lipid class of maize grown at 0 and 100 μM Al

Lipids	Al (μM)	
	0	100
TL	1.81	1.89
PI	1.19	1.67
PC	1.74	2.08
PG	0.43	2.08
PE	1.38	1.47
MGDG	1.30	1.85
DGDG	2.24	2.08

The double bond index was estimated from mol% fatty acid values indicated in Figs. 5 and 6.

27.3 to 8.2 mol%. Specifically, Al significantly increased the levels of monounsaturated 18:1 and polyunsaturated 18:3 of the MGDG (Fig. 6). The general increase in trienoic 18:3 in most lipid classes (PI, PC, PG, MGDG) and consistent increase in the double bound indices may reflect an increase in membrane fluidity. Concurrent with the accumulation of polyunsaturated fatty acids in shoots, is a general decrease in monounsaturated 16:1 and 18:1 and a decrease in saturated 16:0 and 18:0. Activity of desaturases in the glycerolipid pathway forming dienoic and trienoic fatty acids may be increased. The alterations observed suggest modulation in steps in the desaturation pathways, which is required for the increasing of membrane fluidity. Salinity stress has shown to decrease the proportions of triunsaturated fatty acid (16:3 and 18:3) and increased those of saturated fatty acids, mono- and di-unsaturated fatty acids (16:0, 18:0, 18:1 and 18:2) in the leaves of *Brassica napus* (Najine et al., 1995a,b). Alterations in the length and positions of double bonds within the acyl chain of a lipid molecule can confer widely differing properties and suggest that specific proportions of distinct fatty acid classes may be necessary for optimum membrane function (Hazel and Williams, 1990). The PG exhibits the greatest double bound index increase (Table 4). This can be an accurate indication of the magnitude of adjustments in the membrane fatty acid composition and therefore may reflect the extent to which these adjustments can contribute to plant survive under Al stress. PG plays an important role in maintaining protein organization of the photosynthetic membranes and is considered as major lipid class associated with increased cold tolerance in several plant species (Murata, 1983). Aluminum had no significant effect on TL, PL and on SL, but significantly decreased the GL by 87.4% (Table 5). However, Al increased the PI (3.2-fold) and PG (2.5-fold) content but decreased that of PC (Table 6). However, PE shows no alteration in response to Al stress (Table 6). The MGDG and DGDG were significantly decreased to, respectively, 18.5- and 7.9-fold (Table 6). However, Al increased the MGDG/DGDG ratio from 0.80 to 1.86 (Table 6). The absence of alteration of shoot TL may suggest that they are maintained by the activity of enzymes associated with lipid biosynthesis. Environmental stresses and senescence may lead to breakdown of membrane lipids. In example, salinity stress with sodium chloride decreased total lipid content in the leaves, whereas the stress by calcium sulfate caused an increase (Bettaieb et al., 1980). However, salinity stress by calcium chloride reduced the amount of all fatty acids in the leaves of olive tree (Marzouk et al., 1987). The marked decrease in GL observed in this study can be interpreted to show alterations of photosynthetic membranes. This result is consistent with the studies, including several conducted on most environmental factors that leaf membrane lipids show alterations. This may imply increased galactolipases activity or decreased glycolipid biosynthesis. Drought stress decreased the polar lipids in cotton leaves, the glycolipids being more affected than the phospholipids (Pham Thi et al., 1985). The decrease in DGDG content might reduce the stability of the membrane bilayers (Williams, 1988), the activity of membrane bound-proteins (Cooke and Burden, 1990) and the membrane permeability (Schuler et al., 1991). In regard to PL, an increase in PC is reported in plasma membranes from leaves of *Secale cereale* and *Avena sativa* in response to cold acclimation (Uemura and Steponkus, 1994). Moreover, no significant differences are found in MDA concentrations in shoots between treated and control plants (Fig. 4(b)). Hence, we suggest that maize shoots did not suffer from oxidative damages.

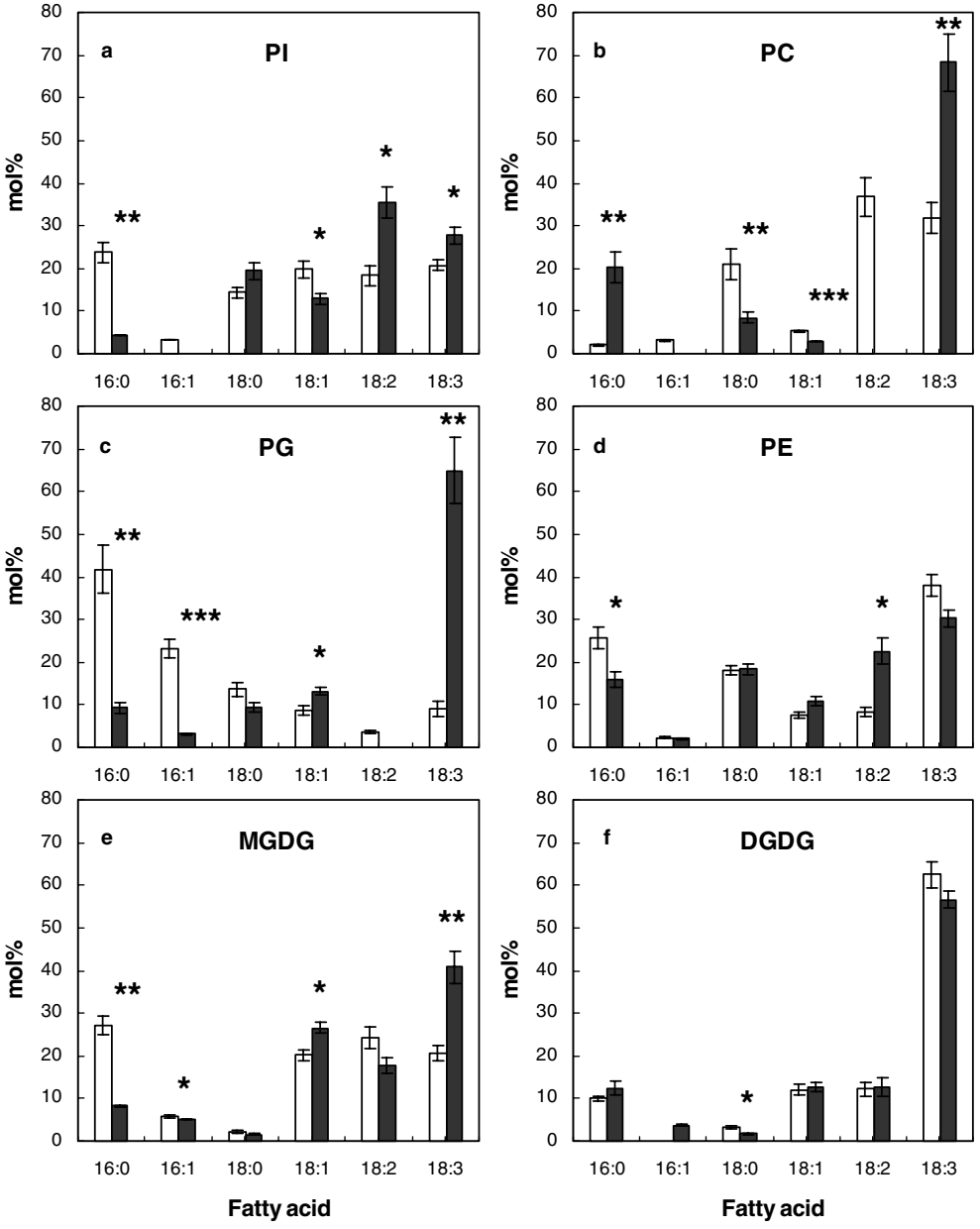

Fig. 6. Effect of Al on fatty acid profiles of various lipid classes obtained from maize shoots. The maize (*Zea mays* L.) seedlings were grown in hydroponics for 8 days then treated with 100 μM Al for 4 days. The histograms presented mean values ± SE obtained from three independent experiments. Significant differences between Al-treated (black bars) and control plants (white bars) were determined using one-way ANOVA: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Table 5
Aluminum effect on lipid classes of maize shoots

Al (μM)	Lipids (mg g^{-1} d. wt.)				SL/PL
	Total lipids	Phospholipids	Glycolipids	Steryl lipids	
0	34.89 ± 1.26	5.51 ± 0.42	17.05 ± 1.16	9.67 ± 1.34	1.75
100	33.28 ± 2.73	5.57 ± 0.17	2.15 ± 0.09***	7.67 ± 0.29	1.38

Values represent means ± SE ($n = 3$). Significant differences between treated and control plants were determined using one-way ANOVA.
*** $P < 0.001$.

Table 6
Aluminum effect on different lipid classes obtained from maize shoots

Al (μM)	Lipid classes (mg g^{-1} d. wt.)						MGDG/DGDG
	PI	PC	PG	PE	MGDG	DGDG	
0	0.92 ± 0.10	3.49 ± 0.72	0.66 ± 0.13	0.44 ± 0.03	7.58 ± 1.83	9.47 ± 1.71	0.80
100	$2.90 \pm 0.41^{**}$	$0.65 \pm 0.24^{*}$	$1.64 \pm 0.08^{**}$	0.38 ± 0.06	$1.40 \pm 0.15^{*}$	$0.75 \pm 0.14^{**}$	1.86

Eight-day-old seedlings were grown in hydroponics and treated with 100 μM Al for 4 days. Values represent means ± SE ($n = 3$). Significant differences between treated and control plants were determined using one-way ANOVA.
* $P < 0.05$.
** $P < 0.01$.

In conclusion, our results show that the changes found in total fatty acids, in the profiles of individual polar lipids, and in the unsaturation levels suggest that membrane structure and function might be altered by Al stress. It should be pointed out that these changes are likely to be attributed to toxicity effects of Al on membrane lipids, rather than the defence responses. These alterations then would be responsible for the morphological and physiological symptoms of Al toxicity. However, Al-induced exudation of citrate, as a basis for mechanism of Al tolerance in maize (Pellet et al., 1995) could have protective effect on membrane lipids. Further research is required to clarify this issue using different maize lines differing in tolerance to Al.

3. Experimental

3.1. Plant material and growth conditions

The corn (*Zea mays* L. cv. Alistrong) seeds were disinfected with 10% (v/v) H_2O_2 for 20 min then rinsed many times with distilled water and germinated in darkness at 25 °C. The seedlings were grown in pots of 12 l capacity containing continuously aerated basal nutrient solution for 4 d. Plants were then transferred homogenously in pots of 6 l each (12 seedlings per pot) for 4 d. The composition of the basal nutrient solution was as follows: (in mM) 2 KNO_3, 2.5 $Ca(NO_3)_2$, 1 KH_2PO_4, 1 $MgSO_4$; (in μM) 50 Fe as Fe–K–EDTA complex, 30 B as H_3BO_3, 10 Mn as $MnSO_4 \cdot H_2O$, 1 Cu as $CuSO_4.5H_2O$, 1 Zn as $ZnSO_4 \cdot 7H_2O$ and 0.2 Mo as $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$. After growing for 8 d, plants were then transferred to nutrient solutions, containing 0 or 100 μM Al added as $Al(NO_3)_3$ and grown for 4 d.

To limit problems of Al speciation and precipitation, the pH was adjusted to 4.0 and checked daily and the Ca^{2+}, Mg^{2+} and $H_2PO_4^-$ concentrations we reduced by half. The seedlings were grown in a controlled-environment chamber with a 16-h light/8-h dark cycle, under 150 μmol m^{-2} s^{-1} light using mercury lamps. The light/dark temperatures were set at 25/22 °C and relative humidity was kept at 65%. After the Al treatment, the roots were washed three times with distilled water. Then, the f. wt. and the d. wt. of roots and shoots were determined.

3.2. Lipid extraction

The lipids were extracted according to the method of Folch et al. (1957) modified by Bligh and Dyer (1959). The plant tissues were washed with boiling water for 5 min to denature phospholipases (Douce, 1964) and then ground in chloroform:methanol mixture (2:1, v/v). The water of fixation was added and the homogenate was centrifuged at 3000 rpm for 15 min. The lower chloroformic phase was aspirated and dried under N_2 stream on a block heater (40 °C) and then, the residue was immediately redissolved in toluene:ethanol (4:1, v/v).

3.3. Fatty acid analysis

The fatty acids in total lipids were separated by thin layer chromatography (TLC) on silica gel G plates 60 (Merck, Darmstadt, Germany) (Sigma). Polar lipids were separated according to the method of Lepage (1969). An aliquot of total lipids was loaded on TLC plates, and developed using chloroform:acetone:methanol:acetic acid:water (50:20:10:10:5, v/v/v/v/v). After they were developed, the plates were moved from the developing tank to a drying tank containing silica gel aerated with N_2. Dried plates were exposed briefly to I_2 vapour and lipid bands were marked with a pencil and the plates were returned to the drying tank to allow the stain to evaporate. The identification of various bands was made by comparison to reference lipids by running authentic standards simultaneously with samples. After TLC, lipid bands were scraped off and placed into glass tubes. Fatty acids from total lipids and lipid classes were transmethylated according to the procedure described by Carreau and Dubacq (1978). An aliquot of lipid extract was evaporated and 2 ml of hexane, a known amount of methyl heptadecanoate (internal standard) and 0.5 ml of 1% sodium methylate were consecutively added. After stirring for 1 min, the sample was allowed to decant for 2 min, then 0.2 ml of 1 N H_2SO_4 and 1.5 ml of distilled water were added. The superior phase that contains fatty acid

methyl esters was aspired and evaporated under N_2 stream. Fatty acid methyl esters were analyzed by GC-FID (HP 6890 series) on a capillary column (HP Innowax, 30 m × 0.25 μm × 250 μm) with a stationary phase made of PEG. The analysis conditions were as follows: carrier gas N_2 at 1.5 ml min^{-1}, oven temperatures isotherm at 150 °C for 1 min, from 150 to 200 °C at the rate of 15 °C min^{-1}, from 200 to 225 °C at the rate of 2 °C min^{-1} and isotherm at 225 °C during 2 min. Injector and detector temperatures were held, respectively, at 250 and 275 °C. Fatty acids were identified by comparison of their retention times to those of standards and the concentrations of individual fatty acids were determined using standard calibration curves. An estimation of the lipid total unsaturation level (double-bond index for C18 fatty acids DBI) was calculated from the mol% values derived from the gas chromatographic data, according to the equation: [∑(mol% 18 C fatty acid content × number of double bonds)]/100 as described by Skoczowski et al. (1994).

3.4. Determination of steryl lipids

Steryl lipids were determined in total lipids according to Huang et al. (1961). An aliquot of lipid extract (200 μl) was evaporated in glass tubes. After addition of 1 ml of acetic acid, the tubes were vortexed and 2 ml of Liebermann-burchard reagent (1 ml of concentrated H_2SO_4 was added to 20 ml of acetic anhydride) were added. The tubes were incubated at room temperature in darkness for 1 h and the absorbance was measured at 525 nm. Cholesterol (Sigma) was used as a standard.

3.5. Estimation of lipid peroxidation

The extent of lipid peroxidation was estimated by measuring the amount of MDA by the method described by Heath and Packer (1968), which takes into account the possible influence of interfering compounds in the assay for thiobarbituric acid (TBA)-reactive materials. The plant tissues were ground in 10% TCA containing 0.25% (w/v) TBA. The samples were heated at 95 °C for 30 min and, after cooling, were centrifuged at 1000*g* for 10 min.

The absorbance of the supernatant was read at 532 nm and corrected by subtracting the non-specific absorbance at 600 nm. The MDA concentration was calculated using molar extinction coefficient of 155 mM^{-1} cm^{-1}.

3.6. Statistical analysis

All data were statistically analyzed using one-way ANOVA, and differences were considered significant at $P < 0.05$.

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